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Increased NAD(P)H oxidase-mediated superoxide production in renovascular hypertension: Evidence for an involvement of protein kinase C

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Increased NAD(P)H oxidase-mediated superoxide production in renovascular hypertension: Evidence for an involvement of protein kinase C.

Background. Angiotensin II infusion has been shown to cause hypertension and endothelial dysfunction and to increase superoxide (O_2^-) production in vascular tissue, mainly via an activation of nicotinamide adenine dinucleotide (phosphate) [NAD(P)H]-dependent oxidase, the most significant O_2^- source in endothelial and/or smooth muscle cells. With these studies, we sought to determine whether endothelial dysfunction in renovascular hypertension is secondary to an activation of these oxidases.

Methods. Endothelial function in aortas from rats with two kidney-one clip (2K-1C) hypertension and age-matched controls was assessed using isometric tension studies in organ chambers. Changes in vascular O_2^- production were measured using lucigenin-enhanced chemiluminescence and electron spin resonance spectroscopy.

Results. In hypertensive animals, relaxation to endothelium-dependent (acetylcholine) and endothelium-independent nitrovasodilators (nitroglycerin) was impaired. Constriction to a direct activator of protein kinase C (PKC) phorbol ester 12,13 dibutyrate (PDBu) was enhanced, and vascular O_2^- was significantly increased compared with controls. Vascular O_2^- was normalized by the PKC inhibitor calphostin C, by the inhibitor of flavin-dependent oxidases, diphenylene iodonium, and recombinant heparin-binding superoxide dismutase, whereas inhibitors of the xanthine oxidase (oxypurinol), nitric oxide synthase (N^G-nitro-L-arginine) and mitochondrial NADH dehydrogenase (rotenone) were ineffective. Studies of vascular homogenates demonstrated that the major source of O_2^- was a NAD(P)H-dependent oxidase. Incubation of intact tissue with PDBu markedly increased O_2^-, the increase being significantly stronger in vessels from hypertensive animals as compared with vessels from controls. Endothelial dysfunction was improved by preincubation of vascular tissue with superoxide dismutase and calphostin C.

Conclusions. We therefore conclude that renovascular hypertension in 2K-1C rats is associated with increased vascular O_2^- leading to impaired vasodilator responses to endogenous and exogenous nitrovasodilators. Increased vascular O_2^- is likely secondary to a PKC-mediated activation of a membrane-associated NAD(P)H-dependent oxidase.

[1] **T.H. and U.W. contributed equally to the manuscript and should both be considered as first authors.**

Key words: arterial hypertension, angiotensin II, phosphorylation, endothelial cell, vasomotor function, nitric oxide, PKC, nitrovasodilators.

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Arterial hypertension represents a major risk factor for the development of atherosclerosis and is associated with abnormal vasomotor function [1]. Experimental [2] as well as clinical studies [3] have demonstrated impaired endothelium-dependent relaxation secondary to decreased activity of the endothelium-derived relaxing factor as one of the basic underlying mechanisms. Endothelium-derived relaxing factor, which is now recognized to be either nitric oxide (NO) [4] or a related compound [5], is destroyed by superoxide (O_2^-)-generating systems [6]. Recent studies have demonstrated that endothelial cells as well as smooth muscle cells posses O_2^--producing enzymes such as nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide phosphate (NADH/NADPH)-dependent oxidases [7, 8]. These oxidases represent the most significant O_2^- source in vascular tissue [7, 8], and the activity of these oxidases is increased *in vitro* upon stimulation with angiotensin II [8]. The mechanisms by which angiotensin II activates the vascular NAD(P)H oxidases is not known. Previous *in vitro* studies have demonstrated that incubation of vascular tissue with direct activators of protein kinase C (PKC) markedly increases steady-state O_2^- production, although the enzymatic O_2^- source was not identified [9]. Constrictions induced by angiotensin II are at least in part mediated by PKC [10], and in neutrophils, the activation of the NAD(P)H oxidase is triggered via a PKC-mediated phosphorylation of the cytosol located p_{47}phox subunit [11]. Recently, we have demonstrated that angiotensin II infusion can cause hypertension, endothelial dysfunction, and increases in vascular O_2^- production secondary to an activation of NAD(P)H-dependent oxidases [12].

To our knowledge, no study has demonstrated that in the setting of two kidney—one clip (2K-1C) hypertension endothelial dysfunction is associated with/and or secondary to increased vascular O_2^- production.

Based on these considerations, we examined the consequences of an activation of the endogenous, circulating renin-angiotensin on vascular O_2^- production, NAD(P)H oxidase activity, and endothelial function in the animal model of renal artery stenosis. We also sought to determine a possible contribution of PKC in mediating the activation of these O_2^--producing enzymes.

METHODS

Animal preparation

Studies were performed in male Sprague-Dawley rats (Kißlegg, Germany). The animals had free access to tap water and standard rat chow. In rats weighing 120 to 140 grams, 2K-1C was induced as recently described [13, 14]. For this purpose, a rigid U-shaped silver clip (I.D. 0.2 to 0.25 mm) was applied on the right renal artery through a loin incision while the rat was under ketamine/xylazin anesthesia. The contralateral kidney remained untouched. Sham-operated rats served as normotensive controls. Hypertensive rats were selected on the basis of systolic blood pressure values obtained in conscious rats by use of tail-cuff plethysmography. All experiments were done eight to nine weeks after clipping.

Vessel preparation and organ chamber experiments

The thoracic aorta of renal hypertensive and age-matched control normotensive rats was isolated after killing and placed into cold buffer solution of the following composition (mM): NaCl 118.3, KCl 4.69, $CaCl_2$ 1.87, $MgSO_4$ 1.20, K_2HPO_4 1.03, $NaHCO_3$ 25.0, and glucose 11.1; pH 7.4). The aorta was cleaned of excessive adventitial tissue and was cut into 5-mm ring segments. Eight ring segments were suspended in individual organ chambers filled with buffer solution (95% O_2, 5% CO_2, 37°C), and changes in isometric tension were recorded with linear force transducers. All experiments were performed in the presence of 10 μM indomethacin to prevent synthesis of prostaglandins. Before the experiments, the rings were gradually stretched and equilibrated at a resting tension of 2 grams for at least one hour. Subsequently, the ring segments were exposed to 80 mM KCl at least three times until a constant response during isometric contraction occurred. The vessels were preconstricted with phenylephrine (10^{-7} m) to achieve the 40% to 50% of maximal KCl-induced tone, and then endothelium-dependent and endothelium-independent vasomotor tones were tested by adding acetylcholine (ACh; 1 nM to 3 μM) and nitroglycerin (0.1 nM to 3 μM) to the tissue bath. After another 45 minutes, incubation concentration response curves for KCl and phorbol ester 12,13-dibutyrate (PDBu; 1 nM to 3 μM) were obtained by a cumulative addition to the tissue bath in one-half log dose increments. Vasoconstrictor responses were expressed in percentage of contraction induced by 80 mM KCl. In separate experiments, the relaxant response to acetylcholine was assessed in the presence or absence of conventional superoxide dismutase (SOD; 200 U/ml) as recently described [15]. To address the role of PKC in mediating endothelial dysfunction, aortic rings from control and hypertensive animals were incubated with the PKC inhibitor calphostin C (50 nM) for 30 minutes. Calphostin C was washed out before testing the acetylcholine dose-response relationship again.

Measurement of O_2^- production in intact vessels

Lucigenin chemiluminescence assay. O_2^- production was measured using lucigenin-enhanced chemiluminescence as described previously [12, 16]. Briefly, after preparation, 5-mm ring segments were placed in a modified Krebs/HEPES buffer and were allowed to equilibrate for 30 minutes at 37°C. Scintillation vials containing 2 ml Krebs/HEPES buffer with 250 μM lucigenin were placed into a scintillation counter switched to the out-of-coincidence mode. After dark adaptation, background counts were recorded, and a vascular segment was added to the vial. Scintillation counts were then recorded every minute for 15 minutes, and the respective background was subtracted. The vessels were then dried for 24 hours in a 90°C oven for expressing results as counts per minute per mg dry weight.

To examine the potential role of PKC as a source of O_2^- in the vessels, isolated vascular segments were exposed to the direct activator of PKC, PDBu (10 μM, 15 min), and to calphostin C (50 nM, 30 min), a potent and specific antagonist of PKC. Incubation was performed under fluorescent light because the inhibitory activity of calphostin C against PKC is light dependent. Furthermore, in some experiments, either diphenylene iodonium (10 μM), oxypurinol (100 μM), rotenone (100 μM), or N^G-nitro-L-arginine (L-NNA; 10 μM) was added to the vessel segments to examine a role for flavin-containing enzymes, xanthine oxidase, mitochondrial respiration, and NO synthase, respectively.

To validate data obtained with high lucigenin concentrations, separate experiments using low concentrations of lucigenin were performed (5 μM) [17].

Electron spin resonance (ESR) spectroscopy. The rate of enzyme-mediated formation of O_2^- in vessels was determined using ESR spectroscopy and a membrane permeable, ESR silent, sterically hindered hydroxylamine spin-trap 1-hydroxy-3-carboxy-2,2,5,5,-tetramethylpyrrolidine (CPH; Alexis Biochemicals, San Diego, CA, USA) to detect the formation of O_2^- as concentration of stable nitroxyl radicals (CP°) due to the reaction between CPH and O_2^- [18].

Probes of vessels for ESR measurements were placed into quartz capillaries (internal diameter 1 mm). Intensities of ESR signals were quantitated measuring magnitudes of low field component of triplet ESR signal of CP° radical using a dual-probe ESR resonator and an ESR calibration probe (Bruker, Karlsruhe, Germany). Vessels of the same mass (approximately 2 mg of dry weight) in a form of strips 0.6 mm × 20 mm were incubated at room temperature with the NO synthase inhibitor L-NMMA (0.01 mM, 30 min) to prevent the quenching of the signal due to endothelial-formed NO. Thereafter, the vessels were incubated for five minutes in Krebs-Henseleit solution (pH 7.4) containing CPH (1 mM) and diethyl-tetrapentaacetic acid (0.1 mM) as a chelator of ions of transition metals. Diethyl-tetrapentaacetic acid was used to decrease the rate of Cu^{2+}-catalyzed oxidation of CPH by oxygen as well as formation of OH^{-} radicals and lipid peroxidation both induced by H_2O_2 in vascular cells in presence of trace amounts of ions of transition metals (Fe^{2+}, Cu^{2+}) in Krebs-Henseleit solution.

Settings of ESR spectrometer (ECS 106; Bruker, Karlsruhe, Germany) were as recently described [18]. Data are given as CP° radicals formed per mg tissue.

Measurement of O_2^{-} production in vessel homogenates

Aortic segments (3 to 4 cm) were homogenized on ice with a glass/glass motor-driven tissue homogenizer for two minutes in phosphate-buffered saline. The homogenate was then centrifuged at 750 grams for five minutes. The pellet was discarded, and the supernatant was stored on ice until use.

NADH or NADPH oxidase activity was measured by chemiluminscence in a scintillation vial containing HEPES buffer, lucigenin, and 100 μM NADH or 100 μM NADPH as the substrate. No activity could be measured in the absence of NADH and NADPH. Reactions were initiated by addition of 25 μl homogenate. To determine the O_2^{-} dependency of the lucigenin-enhanced chemiluminescence obtained from vascular homogenates, we examined the effect of a recombinant heparin-binding chimeric Cu^{2+}/Zn^{2+} SOD (HB-SOD, 20 U/ml) on O_2^{-} production after stimulation of homogenates with NADH. In previous studies, we found that native Cu^{2+}/Zn^{2+} SOD was relatively ineffective at inhibiting the lucigenin signal, whereas HB-SOD was approximately 100-fold more potent in scavenging O_2^{-} generated by homogenates of vascular tissues [16].

In some experiments, membranes and cytosol were separated by centrifugation (50,000 grams for 30 min). Twenty-five microliters of either the supernatant or the particulate fraction (which had been resuspended in 200 μl buffer) were used to examine oxidase-dependent O_2^{-} production of these cellular subfractions. Net chemiluminescence yields were integrated by calculating the area under the curve of chemiluminescence for nine minutes and were converted to nmol O_2^{-}. Calibration of lucigenin chemiluminescence was accomplished using known rates of O_2^{-} production from 0 to 5 mU/ml xanthine oxidase plus 100 μM xanthine, as determined by cytochrome c reduction (E_M 21 · mM^{-1} · cm^{-1}). Values were standardized to the amount of protein present. Protein content was measured using a commercially available kit (Bio Rad Laboratories, Richmond, CA, USA).

Morphometric analysis

Aortic segments (2 to 3 mm in length) were placed in buffered formalin for morphometric evaluation. After dehydration, the segments were longitudinally embedded in paraffin. Paraffin-embedded tissue blocks were sectioned at a thickness of 5 μM and were stained by hematoxylin eosin. Mean media thickness was determined using a commercially available digitizing system.

Data analysis

Results are expressed as mean ± SEM. The ED_{50} value for each experiment was obtained by logit transformation. Comparisons of vascular responses (ED_{50} and maximal percentage relaxation) and O_2^{-} production were performed using analysis of variance. When significance was indicated, a Student-Newman-Keuls post hoc test was used to indicate differences between groups. *P* values of less than 0.05 were considered significant.

RESULTS

Effects of renal artery clipping on blood pressure and media thickness

Renal artery clipping significantly increased mean arterial pressure (4 weeks, 183 ± 5 mm Hg; 8 weeks, 217 ± 7 mm Hg) as compared with normotensive controls (4 weeks, 117 ± 3 mm Hg; 8 weeks, 124 ± 6 mm Hg, $P < 0.05$ for each value). At week nine, medial thickness averaged 77 ± 6 μM in normotensive controls ($N = 5$), which was significantly less compared with medial thickness of aortae from hypertensive animals (103 ± 3 μM; $N = 6$; $P < 0.05$).

Vasorelaxation responses to acetylcholine and nitroglycerin

Acetylcholine relaxed aortas from control rats in a dose-dependent fashion to maximal 96% ± 4% with an ED_{50} of 7.63 ± 0.06 (–log M, $N = 13$). This effect of acetylcholine was significantly depressed in 2K-1C rats ($N = 13$; Fig. 1). The sensitivity [ED_{50} (–log M), 7.19 ± 0.12] and the maximal relaxations (75% ± 3%) were less than those in the control group. Endothelium-independent responses to nitroglycerin were blunted in 2K-1C rats compared with control vessels [ED_{50} (–log M), 7.47 ± 0.07 vs. 7.87 ± 0.13, $P < 0.05$; Fig. 1]. Impaired maximal relaxations to the endothelium-dependent vasodilator acetylcholine were significantly improved by preincubation with conventional SOD (200 U/ml, $N = 6$)

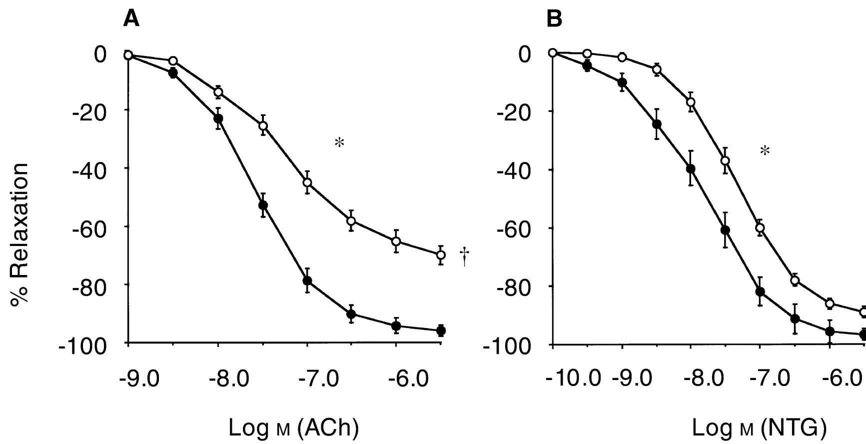

Fig. 1. Endothelium-dependent relaxation to acetylcholine (ACh) and endothelium-independent relaxation to nitroglycerin (NTG) of aortic segments from sham-operated control rats (-●-) and hypertensive two kidney-one clip (2K-1C) animals (-○-). Data are presented as mean ± SEM. *$P < 0.05$ vs. control for ED_{50}. †$P < 0.05$ vs. control for maximal relaxation.

Table 1. Effects of superoxide dismutase (SOD) and protein kinase C (PKC) inhibition with calphostin C (Cal C) on endothelial function in control and hypertensive animals

	Acetylcholine	
	ED_{50} *−log M*	Maximum relaxation *%*
Control	−7.61 ± 0.09	91 ± 3
Control + SOD	−7.56 ± 0.09	94 ± 2
2K-1C	−7.17 ± 0.08[a]	72 ± 4[a]
2K-1C + SOD	−7.23 ± 0.07[a]	90 ± 1[b]
Control	−7.74 ± 0.10	97 ± 2
Control + Cal C	−7.61 ± 0.08	94 ± 1
2K-1C	−7.22 ± 0.06[a]	73 ± 4[a]
2K-1C + Cal C	−7.17 ± 0.09[a]	87 ± 3[ab]

Data are presented as mean ± SEM from 4 to 8 experiments.
[a] $P < 0.05$ vs. control with and without calphostin C (50 nM) or superoxide dismutase (200 U/ml)
[b] $P < 0.05$ vs. two kidney-one clip animals (2K-1C) without Cal C or SOD

and calphostin C (100 nM, $N = 8$; Table 1), whereas incubation of control aortic rings with both drugs did not modify endothelial function.

Vasoconstrictor responses to KCl and PDBu

Vasoconstrictor responses to KCl were comparable in both groups (ED_{50} 16.7 ± 0.5 vs. 23.3 ± 0.9 mM). The sensitivity and maximal contractions to PDBu were significantly increased in hypertensive 2K-1C rats ($N = 9$) compared with the control ($N = 8$) animals [ED_{50} (–log M), 7.60 ± 0.17 vs. 7.12 ± 0.08, respectively, $P < 0.05$; Fig. 2A].

O_2^- production in intact vessels

Relative rates of O_2^- production by vascular segments, as estimated by lucigenin-enhanced chemiluminescence, were increased approximately twofold in 2K-1C hypertensive animals as compared with vessels from controls (Table 2). The PKC inhibitor calphostin C markedly reduced O_2^- while having no effect on O_2^- production in control vessels (Fig. 2B). In contrast, incubation of vascular tissue with PDBu increased O_2^- significantly less in vessels from control animals (from 2.6 ± 0.3 to 29.3 ± 6 counts × 10^3/mg) as compared with vessels from hypertensive animals (4.6 ± 0.4 to 53.2 ± 8 counts × 10^3/mg). Removal of the endothelium decreased O_2^- in vessel segments from both animal groups to a similar extent (Table 2). Incubation with 10 μM diphenylene iodonium for 10 minutes markedly attenuated the lucigenin signal in both control and hypertensive vessels, whereas oxypurinol, L-NNA, and rotenone had no significant effect.

Similar to results obtained with high concentrations of lucigenin (250 μM), experiments with ESR spectroscopy (Fig. 3A) and with low-dose lucigenin (5 μM; Fig. 3B) revealed a twofold to threefold increase in O_2^- in the aortic rings of hypertensive animals compared with controls.

NADH/NADPH activity in vascular homogenates

Superoxide production in vascular homogenates in response to 100 μM NADH or NADPH was significantly higher in aortas from hypertensive animals as compared with controls (NADH, 4.4 ± 0.3 vs. 2.3 ± 0.2 nmol O_2^-/mg/min, $P < 0.05$; NADPH, 2.6 ± 0.0.1 vs. 1.7 ± 0.1 nMol · O_2^-/mg/min, $P < 0.05$). Heparin-binding superoxide dismutase (20 U/ml), a recombinant form of superoxide dismutase, which contains a glycosaminoglycan-binding region [16], was highly effective in reducing the chemiluminescence signal (from 2.4 ± 0.1 to 1.1 ± 0.1 in sham-operated animals and from 4.5 ± 0.2 to 1.2 ± 0.2 in hypertensive 2K-1C rats; Fig. 4).

After separation of cellular subfractions by centrifugation, membrane-bound activity and cytosolic oxidase activity were measured. NADH- and NADPH-driven O_2^- production was predominantly located in the particulate fraction (Fig. 5). Cytosolic activity was minimal. NADH- and NADPH-dependent activity was approximately twofold higher in hypertensive animals compared with control animals.

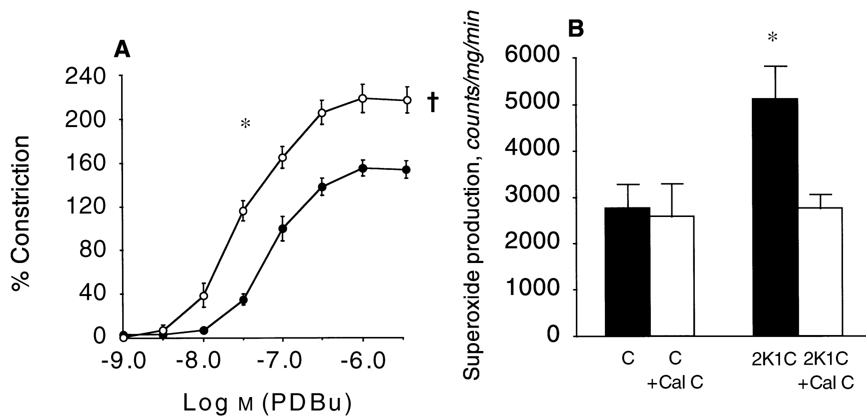

Fig. 2. (*A*) Vascular constriction to phorbol ester 12,13-dibutyrate (PDBu) of aortic rings from control (●) and 2K-1C (○) animals. (*B*) Effect of inhibition of protein kinase C (PKC) by calphostin C on superoxide (O_2^-) production in intact aortic ring segments from both groups. Data are presented as mean ± SEM. (*A*) $*P < 0.05$ in ED_{50} vs. control, †$P < 0.05$ in maximum constriction vs. control. (*B*) $*P < 0.05$ vs. control.

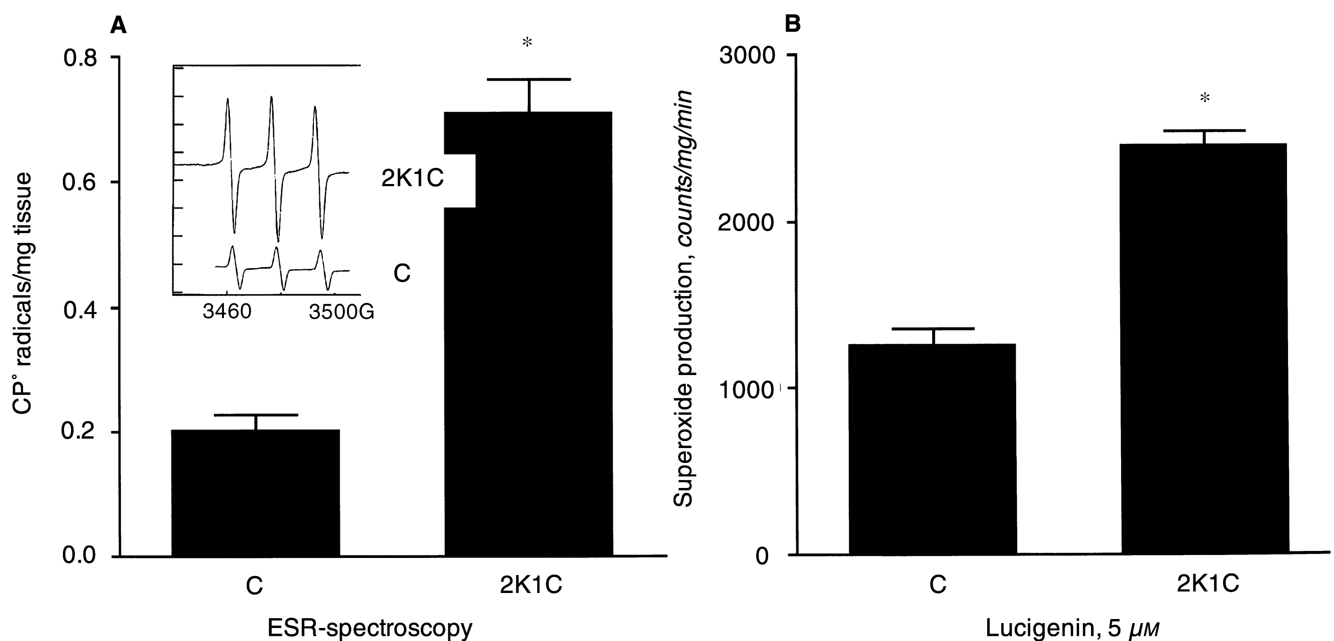

Fig. 3. Intensities of an electron spin resonance spectroscopy (ESR) signal of CP° radicals in aortas of control and 2K-1C animals on the left hand side (*A*) and superoxide (O_2^-) production quantified by lucigenin enhanced chemiluminescence (5 μM) (*B*). Original ESR-spectra of an aorta from control (C) and from hypertensive animal (2K-1C) is shown as an insert. Data are presented as mean ± SEM. $*P < 0.05$ vs. controls.

DISCUSSION

With the present studies, we demonstrate for the first time, to our knowledge, that renovascular hypertension in 2K-1C rats is associated with increased vascular O_2^- leading to impaired vasodilator responses to endogenous and exogenous nitrovasodilators. In studies of vascular homogenates, we identified a membrane-associated, NADH/NADPH-dependent oxidase as the most significant O_2^- source. Vascular O_2^- production was normalized by the addition of the PKC inhibitor calphostin C and by recombinant HB-SOD. The increase in O_2^- production in response to a direct activator of PKC was significantly stronger in vessels from hypertensive animals as compared with controls. Accordingly, PKC inhibition, as well as the addition of conventional SOD, improved endothelial dysfunction in hypertensive animals. We therefore conclude that endothelial dysfunction in renovascular hypertension is secondary to a PKC-mediated increase in the activity of the vascular NADH-/NADPH-driven oxidase.

Our results obtained on the aorta from rats with renovascular hypertension confirm earlier observations that endothelium-dependent relaxations to acetylcholine are markedly depressed [19]. The mechanisms underlying this phenomenon are likely multifactorial and may include deficiencies in the substrate for the ·NO synthase

Table 2. Superoxide production in intact aortic segments, in response to various interventions

Interventions	*N*	Control	2K-1C
Endothelium +	16	2.6 ± 0.3	4.6 ± 0.4[c]
Endothelium −	9	2.1 ± 0.2[b]	3.6 ± 0.3[bc]
Diphenylene iodinium *100 μM*	7	1.3 ± 0.2[a]	1.3 ± 0.3[a]
Oxypurinol *100 μM*	5	2.7 ± 0.4	4.7 ± 0.2
L-NNA *10 μM*	4	2.3 ± 0.3	4.4 ± 0.4
Rotenon *100 μM*	4	2.9 ± 0.4	4.5 ± 0.3

All values are means ± SEM. Superoxide is expressed as counts · 10^3/mg of dry wt of vessels/min.
[a] $P < 0.01$ vs. vessels without interventions
[b] $P < 0.05$ vs. nondenuded vessels
[c] $P < 0.001$ vs. control (statistical analysis only performed on endothelium (+), endothelium (−), and diphenylene iodonium groups

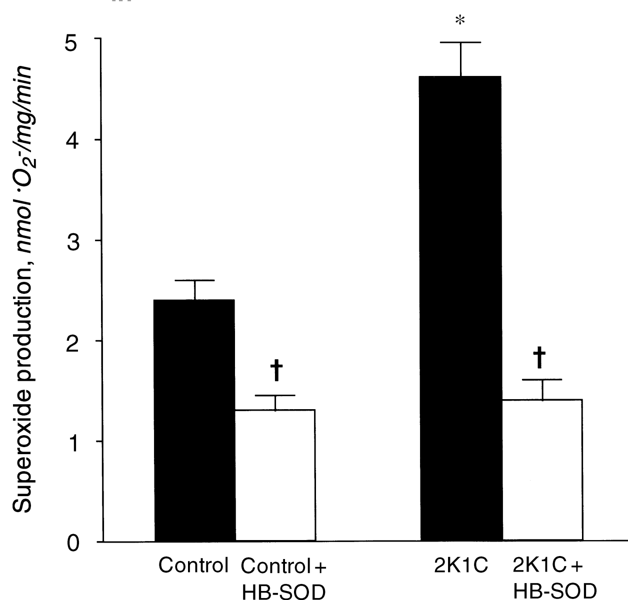

Fig. 4. Effect of recombinant heparin-binding superoxide dismutase (HB-SOD) on superoxide (O_2^-) production after stimulation of vascular homogenate from control and hypertensive 2K-1C animals with NADH. In 2K-1C rats, O_2^- production (NADH-oxidase activity) was significantly increased. HB-SOD inhibited the chemiluminescence signals in both groups to a similar level. Data are presented as mean ± SEM. *$P < 0.05$ vs. control, †$P < 0.05$ vs. without HB-SOD.

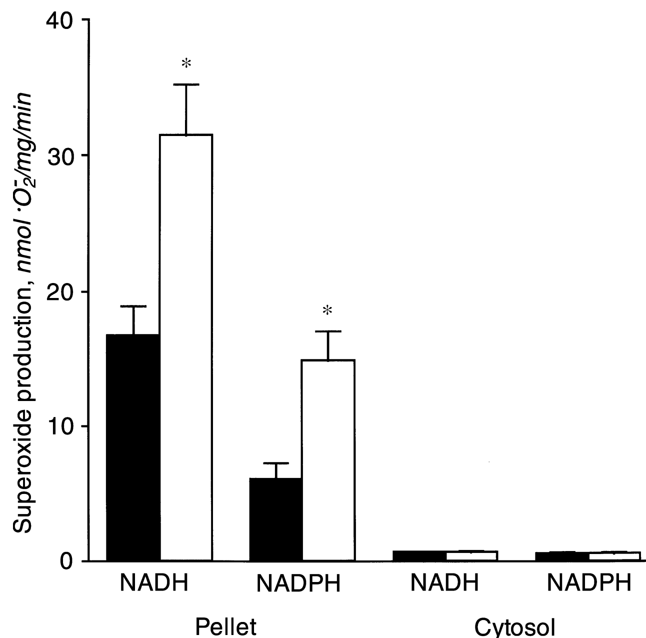

Fig. 5. Subcellular location of NADH and NADPH-oxidase activity in vascular homogenates from control (■) and hypertensive 2K-1C animals (□). Superoxide production in response to NADH and NADPH was increased in the particulate fraction (pellet). Cytosolic superoxide (O_2^-) production was negligible. Data are presented as mean ± SEM. *$P < 0.05$ vs. control.

(L-arginine), decreased activity, and/or expression of the ·NO synthase, an imbalance between the production of endothelium-derived constricting and relaxing factors, and finally an enhanced degradation of ·NO secondary to increased production of oxygen-derived free radicals such as O_2^-. In the setting of renovascular hypertension, two groups independently showed that ·NO production is increased rather than decreased [2, 20]. By using a bioassay technique, Van de Voorde and Leusen observed that the perfusate of an acetylcholine stimulated aorta from a hypertensive rat caused a relaxation of an endothelium denuded detector vessel that was even somewhat stronger than the relaxation effect seen after stimulation of the preparation of a normotensive rat [2]. Indirect evidence for increased ·NO production in vessels from animals with renovascular hypertension was provided from studies showing that the inhibition of ·NO synthase by L-NAME resulted in an exaggerated increase in systemic pressure and vascular resistance in 2K-1C rats compared with normotensive controls [20]. Taken together, these findings strongly suggest that the production of endothelium-derived NO is increased rather than decreased, and point to a potential role of enhanced ·NO breakdown, for example, due to O_2^-.

Renovascular hypertension in the 2K-1C Goldblatt rat model depends on a renin-induced increase in circulating angiotensin II levels [21]. *In vitro* studies have shown that incubation of cultured vascular smooth muscle cells with angiotensin II increases O_2^- mainly via stimulation of NADH-/NADPH-dependent oxidases [8]. More recently, we demonstrated [12] that angiotensin II infusion for a five-day period caused hypertension and endothelial dysfunction associated with twofold to threefold increase in vascular O_2^- secondary to an activation of the NADH/NADPH oxidase. The increase in O_2^- production was angiotensin II specific and not secondary to the hypertensive effects of angiotensin II. This assumption was based on experiments with norepinephrine infusion where a similar degree of hypertension induced by norepinephrine did not modify endothelial function and vas-

cular O_2^- [12]. However, the animals in that previous study were hypertensive for three to four days, whereas in our studies, the hypertensive period averaged seven to eight weeks, making comparisons between these two studies rather difficult.

With our studies, we could demonstrate that 2K-1C renovascular hypertension as an angiotensin II-dependent form of hypertension leads to endothelial dysfunction and to a significant increase in vascular O_2^-. In intact vascular segments, O_2^- production was inhibited using diphenylene iodonium, whereas oxypurinol, rotenone, and L-NNA had no effect. These observations exclude a significant contribution of the xanthine oxidase, the mitochondrial NADH-dehydrogenase and the NO synthase, respectively. The most significant O_2^- source identified was a membrane-associated oxidase producing O_2^- to a greater extent in response to NADH than NADPH. These features are therefore almost identical to prior observations in cultured vascular smooth muscle cells and in the angiotensin II infusion model [8, 12]. Removal of the endothelium reduced O_2^- production to a similar extent in control and hypertensive animals, suggesting that most of the increase in O_2^- in hypertensive rats was derived from vascular smooth muscle. These NADH/NADPH oxidases have been demonstrated to represent the major source of O_2^- in both endothelial cells [7] and vascular smooth muscle [8], and are activated upon stimulation with angiotensin II [8]. Although this vascular oxidase system shares many features of the phagocyte NADPH oxidase, there are important differences. Vascular oxidase activation occurs over a period of hours at continuous low levels of O_2^- (nmol/mg/min) [8], whereas the phagocytic oxidase produces "burst"-like high levels of O_2^- (μmol/mg/min) [22]. The substrate utilization for the neutrophil favors NADPH, whereas vascular tissue favors NADH. Recently, a subunit of the NADPH oxidase, the p_{22}phox protein, was cloned in smooth muscle cells [23]. The expression of the p_{22}phox is accompanied by the presence of a spectrophotometrically identifiable cytochrome (553 nm) and functional NADPH oxidase activity. NADPH oxidase has been demonstrated to regulate angiotensin II-induced hypertrophy in vascular smooth muscle cells [8], and the p_{22}phox expression is up-regulated in angiotensin II-induced hypertension [24]. Stable transfection of antisense p_{22}phox resulted in a significant inhibition of the angiotensin II-induced activation of the NADH as well as the NADPH-dependent O_2^- production [25], suggesting that this oxidase represents one enzyme using NADH as well as NADPH as a substrate for O_2^- production.

The mechanism whereby angiotensin II activates the NADH/NADPH oxidase is still unclear but may involve a PKC-dependent process. With these studies, we could demonstrate that constrictions induced by a direct activator of PKC, PDBu, were markedly enhanced in vessels from hypertensive animals as compared with controls indicating increased vascular PKC activity. Incubation of intact vessels with a PKC inhibitor such as calphostin C had no significant effect on O_2^- in controls, whereas increased O_2^- production of hypertensive animals was markedly reduced. These observations go along with studies from neutrophils, in which stimulation with phorbol ester has been shown to lead to a phosphorylation of the cytosolic p_{47}phox subunit, which in turn triggers assembly and subsequent activation of the NADPH oxidase [26] and in which inhibition of PKC resulted in an inhibition of O_2^- production [27]. This concept is further strengthened by our observation that PDBu increased vascular O_2^- and that the observed increase in O_2^- was markedly stronger in vessels from hypertensive as compared with vessels from control animals. In addition, preincubation with calphostin C partially improved endothelial dysfunction in hypertensive animals while having no significant effect on acetylcholine responses in control vessels. Based on these findings, it is conceivable to conclude that at least part of the increased O_2^- and endothelial dysfunction in aortic rings from hypertensive animals is secondary to PKC activation.

The vast majority of the data reported to quantitate O_2^- formation in vessels and vessel homogenates are based on the measurements of intensity of lucigenin-enhanced chemiluminescence [12, 16, 28]. Vessels were usually incubated with high lucigenin concentrations of approximately 250 μM in order to obtain enough sensitivity. The validity of these data was recently questioned due to observations that lucigenin itself increases formation of O_2^- in the presence of enzymes that are capable to provide a one-electron reduction of lucigenin $^{2+}$ such as NO synthase and the xanthine/xanthine-oxidase system [29, 30]. Based on this background, we performed additional experiments using low concentrations of lucigenin (5 μM), which have been recently shown to cause no additional O_2^- formation [17]. In addition, to measure independently of lucigenin vascular O_2^- in tissue from control and hypertensive rats, ESR spectroscopy was employed using a spin trap, which has been recently described to detect O_2^- [18] with a high sensitivity. With both approaches, we found consistently a twofold to threefold increase in vascular O_2^- in vessels from hypertensives compared with vessels from control animals.

In previous studies, *in vitro* or *in vivo* treatment with different SOD preparations was able to normalize endothelial dysfunction [12] and to prevent the increase in blood pressure [31] in the angiotensin II infusion model and also to prevent the accumulation of O_2^- in the vessel wall [31]. With these studies, we observed a similar phenomenon. Incubation of aortic rings from hypertensive animals with conventional SOD (200 U/ml) significantly improved endothelial dysfunction, whereas it was ineffective in aortic tissue from controls. These observations

suggest that angiotensin II-stimulated production of oxygen-derived free radicals may trigger the development of hypertension presumably via inactivation of NO. We therefore postulate that increased vascular O_2^- production secondary to an activation of vascular NAD(P)H oxidase is at least in part responsible for the observed impaired NO-mediated relaxation, which in turn may contribute to the hypertension seen in this model of renovascular hypertension.

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APPENDIX

Abbreviations used in this article are: ACh, acetylcholine; CP°, stable nitroxyl radicals; CPH, 1-hydroxy-3-carboxy-2,2,5,5,-tetramethylpyrrolidine; DPI, diphenylene iodinum; DTPA, diethyl-tetrapentaacetic acid; EDRF, endothelium-derived relaxing factor; ESR, electron spin resonance; HB-SOD, heparin-binding superoxide dismutase; 2K-1C, two kidney one clip; L-NNA, N^G-nitro-L-arginine; NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; O_2^-, superoxide; PDBu, phorbol ester 12,13-dibutyrate; PKC, protein kinase C; SOD, superoxide dismutase.

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